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Dichotomous effects of aryl hydrocarbon receptor (AHR) activation on human fetoplacental endothelial cell function

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Abstract

Introduction—Maternal cigarette smoking is associated with elevated fetoplacental vascular resistance and fetal growth restriction (FGR). While studies have demonstrated varying effects of nicotine on blood flow, the role of polycyclic aromatic hydrocarbons (PAHs), abundant toxins in cigarette smoke that cross the placenta, has not been elucidated. We hypothesized that exposure of human fetoplacental endothelial cells (ECs) to the PAH benzo[a]pyrene (BaP) would result in up-regulation of cyclooxygenase-2 (PTGS2) and preferential production of vasoconstrictive prostanoids via activation of the aryl hydrocarbon receptor (AHR) pathway.

Methods—ECs were isolated, cultured, and treated with vehicle or BaP. ECs were subjected to real-time PCR, western blotting, enzyme immunoassays, wound scratch assays, tube formation assays, and RNA interference against AHR. Statistical analyses were performed with Student's t-test, one-way ANOVA followed by multiple comparisons testing when appropriate, or the Kruskal-Wallis H test.

Results—BaP induced PTGS2 expression ($p < 0.05$) and production of the stable metabolite of prostacyclin ($p = 0.001$) in fetoplacental ECs without affecting thromboxane. These effects were ablated by PTGS2 inhibition ($p < 0.01$) and RNA interference of AHR ($p < 0.001$). Surprisingly, despite the induction of prostacyclin, EC migration ($p = 0.007$) and tube formation ($p = 0.003$) were inhibited by BaP. AHR inhibition, however, rescued tube formation ($p = 0.008$).

Discussion—BaP-mediated AHR activation results in induction of PTGS2 expression and enhanced production of prostacyclin metabolite. Despite an increase in this vasodilatory and pro-angiogenic prostanoid, BaP exposure also impairs EC migration and angiogenesis through AHR.

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This suggests that PAH may adversely affect the fetoplacental vasculature through its regulation of angiogenesis.

Introduction

Cigarette smoking in pregnancy leads to higher fetoplacental vascular resistance, and this impedance of blood flow contributes to fetal growth restriction (FGR).^{1–4} However, the exact mechanisms by which tobacco use impairs fetoplacental blood flow remain unknown. Nicotine, often considered a central culprit in adverse effects of smoking, has been found to elicit variable effects on blood flow.^{5–7} In contrast, exposure to actual cigarette smoke, which contains a few thousand chemicals, impairs fetoplacental blood flow as demonstrated by abnormal umbilical artery Doppler indices.^{2–4} This suggests that other compounds within cigarettes beyond just nicotine may contribute to altered fetoplacental vascular resistance and FGR associated with smoking during pregnancy.

Polycyclic aromatic hydrocarbons (PAHs) are key toxins present within cigarette smoke condensate and have been implicated in vascular dysfunction. Benzo[a]pyrene (BaP), a classic PAH, is one of the most toxic.⁸ BaP crosses the placenta and has been found in both neonatal tissues and umbilical cord blood after maternal cigarette smoking.^{9–11} In both animal models and human studies, *in utero* exposure to BaP increases risks for a variety of consequences including reduced vascularization of the fetoplacental tree and FGR.^{12–14} One major mechanism by which BaP acts is via activation of the aryl hydrocarbon receptor (AHR) pathway.¹⁵ AHR is a member of the basic helix-loop-helix family of transcription factors and is activated by ligands including BaP. Upon ligand binding to AHR, dimerization of AHR to the aryl hydrocarbon receptor nuclear translocator (ARNT) occurs.^{16,17} The AHR/ARNT heterodimer then binds to xenobiotic response elements in the promoters of key genes, resulting in gene transcription.¹⁸

Proper AHR signaling has several important physiologic consequences. For example, regression of fetal vascular structures does not occur in *Ahr* null mice.^{19–21} Specifically, the ductus venosus remains persistently patent in these mice, whereas closure occurs after *Ahr* activation in mice that were hypomorphic for the *Ahr* allele.^{19–21} These findings, in conjunction with that of deficient fetoplacental vascularization in BaP-exposed mice, implicate *Ahr* signaling on fetoplacental vascular development and fetal growth.^{13,14,19–21}

The specific mechanisms underlying AHR-mediated regulation of vascular function both within and beyond the placenta have not been fully elucidated. AHR activation has been shown to result in cyclooxygenase-2 (PTGS2) induction in several cell and tissue types.^{22,23} Furthermore, other studies suggest that proper closure of fetal vascular structures including the ductus venosus are mediated by prostaglandin production downstream of PTGS2.^{24–26} Thus, the main objective of this study was to determine the effects of AHR activation on PTGS2 expression and prostaglandin biosynthesis within a model of human fetoplacental endothelial cells (ECs). Using BaP as a representative PAH within cigarette smoke and as a ligand of AHR, we hypothesized that BaP-mediated AHR signaling results in preferential production of cyclooxygenase-induced vasoconstrictive prostaglandins, thereby contributing to cigarette smoking-associated aberrant fetoplacental vascular resistance.

Methods

Cell isolation and culture

Human placental villous EC isolation was performed as previously described with minor modifications after approval by the institutional review board at Northwestern University and subject consent.²⁷ Cells were isolated immediately after delivery from placentas from uncomplicated, full-term pregnancies without a personal history of cigarette smoking or tobacco use during pregnancy. None of the subjects were exposed to aspirin or other non-steroidal medications throughout pregnancy. Based on previous data, primary cells were used only through the fifth passage to avoid changes in phenotype.

Cells were cultured with media that was supplemented with 5% fetal bovine serum, bovine brain extract with heparin, epidermal growth factor, hydrocortisone, and gentamicin/amphotericin B (Lonza, Walkersville, MD). Once 70% confluence was achieved, cells were treated for 24 hours with vehicle (dimethyl sulfoxide [DMSO] 1:1000; Sigma-Aldrich, St. Louis, MO) or BaP (10^{-8} M, or 10^{-6} M; Sigma-Aldrich). These concentrations were chosen to correspond to prior data demonstrating BaP concentrations and BaP-DNA adducts in umbilical cord blood of neonates born to smoking mothers.^{9,11} In experiments where specific PTGS2-inhibition was performed, cells were first pre-treated with vehicle or N-[2-(Cyclohexyloxy)-4-nitrophenyl] methanesulfonamide (NS398) for four hours prior to BaP exposure.

RNA isolation and real-time PCR

Total RNA from primary EC cultures was extracted using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). One microgram of RNA was reverse-transcribed using Q-script (Quanta Biosciences; Gaithersburg, MD). Specific oligodeoxynucleotide primers were synthesized according to published information for cDNA of the various genes as previously published, with each primer set spanning two exons, or purchased from Qiagen.²⁸ Primer sets for the constitutively expressed ribosomal protein, large, P0 (*RPLP0*) housekeeping gene were also used.²⁹ Real-time quantitative PCR was used to determine the relative amounts of each transcript using the DNA-binding dye SYBR green (Applied Biosystems, Foster City, CA), and the ABI Prism 7900 HT Detection System (Applied Biosystems). Cycling conditions and cycle threshold analysis were performed as previously described.^{27,28,30}

Protein isolation and immunoblotting

Placental ECs were lysed, and protein concentrations were determined as previously described.²⁷ Equal concentrations of total protein were loaded in each well. Samples were subjected to SDS-PAGE (Invitrogen, Carlsbad, CA) and transferred onto polyvinylidene difluoride membranes (Pall Life Sciences, Port Washington, NY). The following antibodies were used for immunoblotting: polyclonal antibody against PTGS2 (1:1000; Cell Signaling Technology, Danvers, MA), monoclonal antibody against AHR (1:1000; Santa Cruz, Dallas, TX) and monoclonal antibody against beta-actin (ACTB; 1:10,000; Sigma-Aldrich). An antibody directed against Proliferating Cell Nuclear Antigen (PCNA; 1:5000; Cell Signaling) and Caspase-3 antibody (1:2000; Cell Signaling) were utilized to assess effects of BaP on proliferation and apoptosis, respectively. Anti-rabbit and anti-mouse IgG conjugated

to horseradish peroxidase (Cell Signaling Technology) were used as secondary antibodies. Lastly, immunoreactive bands were visualized using an enhanced chemiluminescence detection system (GE Healthcare, Piscataway, NJ).

RNA interference

An RNA oligonucleotide directed against *AHR* and a mismatch negative control small interfering RNA (siRNA) were purchased from Ambion Life technologies (Grand Island, NY). Placental ECs were cultured in media without antibiotics and achieved 50% confluence prior to transfection. On the day of transfection, the RNAiMAX lipofectamine-based reagent (Life technologies) was combined in conjunction with 20 nM siRNA duplexes that were diluted in Opti-Mem I (Life technologies) and applied to the cells. Six hours later, complete growth medium without antibiotics was added, and cells were allowed to recover overnight. The next morning, cells were treated with vehicle or BaP (10^{-6} M) for 24 hours.

Enzyme immunoassay

Placental ECs were treated with vehicle and BaP in the presence and absence of NS398 pre-treatment or were subjected to RNA interference as described earlier. After 24 hours of treatment, culture medium was removed and cells were suspended in serum- and supplement-starved basal media. Twenty-four hours later, cell culture supernatant was collected, centrifuged to remove any cellular contaminants, and transferred to a fresh tube. Enzyme immunoassays (EIAs) were performed for thromboxane B2 (TXB2; main metabolite of thromboxane A2 [TXA2]) and 6-keto-prostaglandin F1 alpha (6-keto-PGF1 α ; main metabolite of prostacyclin [PGI2]) as previously described.³¹ Concentrations were normalized to total protein concentrations.

Tube Formation Assays

Tube formation assays were conducted on growth factor-reduced Matrigel (BD Biosciences), which was added in a volume of 50 μ L/well to a 96-well plate and allowed to polymerize at 37°C for 30 minutes. After polymerization, ECs were stained with calcein AM and plated on the Matrigel at 1.5×10^4 cells/well in 200 μ L of medium.³² Tube formation was observed under an inverted microscope (Zeiss Axiovert 40 CFL) after 18 hours. Images were captured with a Zeiss AxioCam camera attached to the microscope. The tube formation was quantified by measuring the long axis of the individual cells on Matrigel using ImageJ (NIH public domain). Mean values of total length in each sample were used to represent the tube formation.

Wound scratch assays

Placental ECs were plated in equal number (1.5×10^6 cells per well) in 6-well plates and allowed to grow to confluence. A wound was created in each well by scratching the confluent monolayer with a 200 mL pipette tip. Cells were then washed with PBS once followed by replacement of fresh media with vehicle or treatment as indicated. Cells were imaged immediately after wounding. They were imaged again at 6 hours and 24 hours after wounding. ImageJ software was utilized to determine the percentage of wound closure.

Statistical analysis

All cellular experiments were performed on samples isolated from four separate subjects, with each repeated in triplicate. The data from all experiments were pooled and assessed for normality via the Shapiro-Wilk test. Numeric data are reported as means of the three replicates performed in all subjects, with error bars that represent SEM. Statistical analysis for comparison of treatment groups was performed using Student's t-test or one-way ANOVA followed by Scheffe multiple comparison test when appropriate. For non-parametric data, the Kruskal-Wallis H test was utilized. A p-value of <0.05 was considered significant.

Results

BaP induces PTGS2 expression

Within our model of fetoplacental ECs, physiologically relevant doses of BaP induced *PTGS2* mRNA expression in a dose-dependent fashion (overall $p < 0.0001$ for one-way ANOVA; 1.45-fold induction for BaP 10^{-8} M [95% CI 1.05 – 1.85; $p = 0.02$]; 2.80-fold induction for BaP 10^{-6} M [95% CI 2.35 – 3.25; $p < 0.001$]). There was no effect of BaP on cyclooxygenase-1 (*PTGS1*), prostacyclin synthase (*PTGIS*), or thromboxane synthase (*TBXAS1*) expression (Figure 1A). Western blot results demonstrated a trend toward induction of PTGS2 protein expression with BaP 10^{-8} M (2.26 fold-change [95% CI 0.75 – 3.76; $p = 0.06$]). BaP 10^{-6} M, however, led to a statistically significant increase in PTGS2 protein expression (3.09 fold-change [95% CI 1.46 – 4.71; $p = 0.008$]) (Figure 2B, 2C). In order to confirm AHR activation in our model, we also investigated BaP effects on known AHR target genes such as the cytochrome P450 genes *CYP1A1* and *CYP1B1*. We found a significant, dose-dependent induction in both genes ($\chi^2 = 12.9$, $p = 0.012$) (Supplemental Figure 1A). Similarly, dioxin, another AHR ligand was also utilized to demonstrate AHR activation. This also led to induction of *CYP1A1*, *CYP1B1*, in addition to *PTGS2* within our model ($\chi^2 = 13.0$, $p = 0.011$) (Supplemental Figure 1B).

BaP induction of COX-2 is mediated through AHR

We first investigated whether BaP affected *AHR* expression itself, and real-time PCR confirmed no effect (Figure 2A). Similarly, exposure did not influence mRNA expression of *ARNT*, the heterodimeric partner to AHR (Figure 2B). In order to determine whether BaP induction of PTGS2 was indeed occurring via AHR, we performed RNA interference of *AHR*. After exposure of fetoplacental ECs to the lipid transfection reagent, approximately 85–90% of cells were viable in comparison to cells not exposed to lipid reagent based upon Trypan blue dye exclusion (data not shown). This degree of viability did not change regardless of addition of control siRNA or *AHR* siRNA.

As expected cells transfected with control siRNA continued to demonstrate statistically significant induction of PTGS2 (overall $p < 0.0001$ for one-way ANOVA; 1.39-fold induction in comparison to vehicle treatment with control siRNA transfection [95% CI 1.31 – 1.46; $p < 0.001$]). With RNA interference of *AHR*, however, there was complete ablation of *AHR* expression (Figure 2C) and PTGS2 expression was significantly suppressed (0.63 fold-

change in comparison to BaP treatment with control siRNA transfection [95% CI 0.53 – 0.74; $p=0.007$]) (Figure 2D).

6-keto PGF1 α concentrations are increased by BaP treatment

We initially hypothesized that BaP would also up-regulate *TBXAS1* in addition to *PTGS2*, and that this would result in preferential production of the vasoconstrictive prostanoid thromboxane A2 (TXA2). However, because BaP did not affect *PTGIS* or *TBXAS1* expression (Figure 1A), we subsequently speculated that BaP would result in an increase in *both* TXA2 and the vasodilatory prostanoid prostacyclin (PGI2) secondary to *PTS2*-mediated increased production of prostaglandin H2 (PGH2) intermediate. To test this hypothesis, we performed EIAs for thromboxane B2 (TXB2), the stable metabolite of TXA2, along with 6-keto PGF1 α , the stable metabolite of PGI2. We found that TXB2 levels were repeatedly undetectable in cell culture supernatant regardless of vehicle or varying BaP concentrations (data not shown). In contrast, 6-keto PGF1 α levels were significantly increased with BaP treatment (overall $p<0.0001$ for one-way ANOVA; $p=0.001$ when comparing BaP 10^{-6} M treatment to vehicle treatment) (Figure 3A).

BaP-mediated increases in 6-keto PGF1 α are AHR and PTGS2-dependent

In order to determine whether the increase in 6-keto PGF1 α concentrations after exposure to BaP was specifically the result of *PTGS2* induction, we pre-treated cells with NS398, a selective inhibitor of *PTGS2* activity.³³ NS398 did not affect *PTGS2* mRNA (overall $p=0.08$ for one-way ANOVA) or protein expression (overall $p=0.07$ for one-way ANOVA) (Figure 3B, 3C, 3D). However, 6-keto PGF1 α concentrations were significantly suppressed after NS398 pre-treatment (overall $p<0.0001$ for one-way ANOVA; $p<0.01$ for both BaP concentrations before and after NS398 pre-treatment) (Figure 3A). Similarly, RNA interference of *AHR* resulted in significant inhibition of 6-keto PGF1 α concentrations when compared to BaP-treated cells transfected with control siRNA (overall $p<0.0001$ for one-way ANOVA; $p<0.001$ when comparing BaP treatment in control siRNA versus *AHR* siRNA conditions) (Figure 3E).

Fetoplacental EC tube formation is inhibited in the setting of BaP exposure

Our findings of AHR-mediated induction of *PTGS2* expression and production of 6-keto PGF1 α , a known vasodilator, not only contradicted our hypothesis that BaP treatment would result in preferential production of vasoconstrictive prostanoids but also did not correspond with existing literature demonstrating elevated fetoplacental vascular resistance in mice exposed to PAHs. The placentas of these mice, however, were thought to demonstrate increased vascular resistance secondary to an increase in fetoplacental vessel tortuosity and to decreased numbers of placental arteries and arteriole.^{13,14} Thus, we examined whether BaP would negatively affect development of the placental vasculature using EC tube formation angiogenesis assays. In the setting of BaP treatment, tube formation appeared disrupted in comparison to vehicle-treated cells, with a decrease in total tube length noted ($p=0.003$) (Figure 4A, 4B). This finding of disrupted tube formation persisted in cells that were subjected to control siRNA transfection (overall $p<0.0001$ for one-way ANOVA; $p=0.01$ for this post-hoc comparison) (Figure 4C, 4D). In contrast, *AHR* ablation rescued total tube length even in the setting of BaP exposure ($p=0.008$ for this post-hoc comparison)

(Figure 4C, 4D), suggesting that BaP activation of AHR contributes to impaired angiogenesis.

Deficient EC tube formation is the result of impaired EC migration

To determine the mechanistic cellular defect underlying impaired EC tube formation, we evaluated the effects of BaP on various factors, including EC proliferation, apoptosis, and migration. Western blot for PCNA and caspase-3, which were probed on two separate membranes, demonstrated no differences in proliferation or apoptosis, respectively (Figure 5A). In contrast, wound scratch assays demonstrated a deficiency in EC migration at 6 hours and 24 hours ($\chi^2 = 15.9$, $p=0.007$) (Figures 5B, 5C).

Discussion

In this study, we found that exposure of human fetoplacental ECs to physiologically relevant doses of BaP, an abundant PAH in cigarette smoke, led to induction of PTGS2 without affecting *PTGS1*, *PTGIS*, or *TBXAS1* expression. We anticipated that induction of PTGS2 would increase PGH2 substrate availability for uptake and conversion to vascular prostanoids by these prostanoid synthases. However, despite expression of *TBXAS1*, TXB2 levels were consistently undetectable. Instead, we found a preferential increase in 6-keto PGF1 α levels in the setting of BaP treatment while also finding that BaP inhibited EC tube formation via impairment of EC migration.

One potential explanation for our unexpected findings is that PTGS2 preferentially couples with PTGIS within cultured ECs to result in enhanced 6-keto PGF1 α production.^{34,35} It is also possible that other vasoactive mediators are involved. However, upon investigating other potential key players, BaP treatment did not alter expression levels of endothelial nitric oxide synthase (*NOS3*) or endothelin-1 (*EDNI*) (data not shown). Still another possibility is that within our single-cell culture system, we are missing potentially important paracrine interactions. For instance, transcellular production of prostanoids and eicosanoids from PGH2 has been described.^{36,37} Similarly, trophoblast also express AHR, and crosstalk between EC- and trophoblast-derived proteins such as PTGS2 or other metabolizing enzymes may occur.^{38,39} Finally, this increase in vasodilatory prostanoid may represent a compensatory mechanism in the setting of impaired angiogenesis. This possibility is supported by data from others that fetoplacental vessels appeared enlarged in PAH-exposed dams despite reduction in placental arterial vascular volume.¹³

Beyond its vasodilatory capacity, PGI2 enhances angiogenesis through its promotion of EC migration^{40,41} Within our fetoplacental EC model, however, BaP-mediated AHR activation both up-regulates 6-keto PGF 1 α while impairing EC migration and tube formation. This suggests that this disruption in angiogenesis is occurring through non-PGI2-mediated mechanisms and that the increase in PGI2 concentrations is inadequate to rescue the deficiency in angiogenesis. Instead, it is possible that BaP may act via AHR to affect other aspects of angiogenesis. For example, BaP exposure inhibits expression of key subunits of integrin, which are required for vessel stabilization through EC-EC interaction and EC interaction/stabilization with the extracellular microenvironment.^{42,43}

Although this is the first study to our knowledge that aims to identify the mechanistic underpinnings of BaP-mediated AHR activation in human fetoplacental ECs, there are limitations to our study. First, we examined only one of the few thousand chemicals found in cigarette smoke, and it is possible that other toxins may act upon the fetoplacental endothelium differently. Second, in order to isolate the effects of AHR activation, our ECs were obtained from uncomplicated, full-term pregnancies and not from women who smoked and had a growth-restricted fetus. Finally, although we chose physiologically relevant doses of BaP based upon known BaP concentrations and BaP-DNA adducts in umbilical cord blood of neonates born to smoking mothers, we only examined a discrete 24-hour treatment period and were unable to ascertain the effects of chronic BaP exposure.

In summary, we have shown that BaP acts via AHR to induce PTGS2 expression and production of 6-keto PGF1 α . Despite an increase in production of this vasodilatory and pro-angiogenic prostanoid, we also found that BaP impairs EC migration and angiogenesis, which is consistent with murine data that maternal exposure to BaP results in deficient fetoplacental vascularization. These findings are important because despite irrefutable evidence that cigarette smoking during pregnancy remains the leading preventable cause of FGR in developed countries, clinical data are clear that neither smoking cessation counseling nor nicotine replacement therapy during pregnancy significantly reduces smoking or promotes abstinence.^{44,45} Thus, elucidating the mechanisms by which maternal smoking results in placental dysfunction and FGR may yield alternative methods of improving perinatal outcome despite *in utero* exposure to cigarette smoke and its related toxins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Benzo[a]pyrene (BaP) induces cyclooxygenase-2 and increases 6-keto PGF1 α
- Fetoplacental endothelial cell migration and tube formation are inhibited by BaP
- BaP effects on fetoplacental endothelial cells occur via aryl hydrocarbon receptor
- Angiogenic impairment mediates adverse effects of BaP on fetoplacental vasculature

Figure 1.

BaP induces *PTGS2* expression with no significant effect on *PTGS1*, *PTGIS*, or *TBXAS1*. (A) Real-time PCR demonstrates a statistically significant induction of *PTGS2* mRNA in a dose-dependent manner (* $p=0.02$ in comparison to vehicle; $^{\wedge}p<0.001$ in comparison to vehicle). (B) A representative western blot from one subject also demonstrates a BaP-mediated increase in *PTGS2* protein expression. (C) Densitometric analysis of western blot results from all subjects confirms an induction of *PTGS2* protein expression with BaP 10⁻⁶ M ($^{\#}p=0.008$ in comparison to vehicle) and a trend toward induction with BaP 10⁻⁸ M ($p=0.06$).

Figure 2.

Effects of BaP on PTGS2 are mediated through AHR. (A) There was no effect of BaP on *AHR* mRNA expression. (B) Similarly, BaP exposure did not influence *ARNT* mRNA expression. (C) A representative western blot demonstrates complete ablation of AHR expression with transfection of *AHR* siRNA. (D) Densitometric analysis of PTGS2 western blots from all subjects demonstrates up-regulation of PTGS2 protein expression with BaP treatment in the setting of control siRNA transfection (* $p < 0.001$ in comparison to control siRNA and vehicle). With *AHR* ablation, however, BaP treatment resulted in significant suppression of PTGS2 expression ($^{\wedge}p = 0.007$ in comparison to control siRNA and BaP treatment).

Figure 3.

BaP does not affect TXB2 but leads to an induction of 6-keto PGF1 α via the AHR/PTGS2 pathway. (A) EIA demonstrates that BaP increases 6-keto PGF1 α concentrations in a dose-dependent manner, but this is inhibited in the setting of selective PTGS2 inhibition with NS398 (* $p=0.001$ in comparison to vehicle; [^] $p=0.007$ in comparison to BaP 10⁻⁸ M treatment; [#] $p<0.001$ in comparison to BaP 10⁻⁶ M treatment). (B) Real-time PCR shows that BaP-mediated induction of *PTGS2* mRNA expression is not inhibited with use of NS398, a selective inhibitor of PTGS2 activity (overall $p=0.08$ for one-way ANOVA). (C) A representative western blot from one subject also shows that PTGS2 protein expression is not inhibited by NS398 pre-treatment. (D) Graphical representation of western blot analyses from all subjects also confirms no effect of NS398 on PTGS2 protein expression (overall $p=0.07$ for one-way ANOVA). (E) *AHR* silencing via siRNA results in significant suppression of 6-keto PGF1 α concentrations (* $p<0.001$ in comparison to control siRNA and vehicle; [^] $p<0.001$ in comparison to control siRNA and BaP).

Figure 4.

BaP exposure results in impaired EC tube formation. (A) Representative tube formation images of vehicle- and BaP-treated endothelial cells. (B) Graphical representation demonstrates that total tube length is diminished in the setting of BaP treatment (* $p=0.003$). (C) Representative tube formation images in the setting of control siRNA and AhR siRNA transfections demonstrate that BaP-mediated effects on tube formation is mediated via AhR. (D) Graphical representation of total tube length shows that BaP treatment results in impaired angiogenesis in comparison to vehicle treatment in the setting of control siRNA transfection ($^{\wedge}p=0.01$). In contrast, when *AHR* is knocked down, tube formation is rescued in the presence of BaP ($^{\#}p=0.008$).

Figure 5. Deficient EC tube formation is the result of impaired EC migration. (A) Representative western blots shows no difference in proliferation or apoptosis with varying concentrations of BaP treatment in all four subjects. (B) Representative wound scratch images from one subject demonstrate impaired migration after BaP treatment. (C) Graphical representation of wound scratch assays performed on all subjects ($\chi^2 = 15.9$, $p=0.007$).